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Antioxidant activity of chemical constituents from *Prunus avium* seeds

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ABSTRACT

Prunus avium (PA) seeds oil was extracted with pet. ether in a Soxhlet for two days to give an oily residue and analyzed by GC/MS. 24 compounds were detected and identified in which γ-sitosterol (18.57%), erucylamide(12.36%)and are the main. A part from oily residue was treated with hot acetone to precipitate the acetone insoluble constituents in which di 2-ethylhexyl phthalate and octadecanol are main and acetone soluble fraction which saponified to afford the unsaponifiable materials and fatty acids which were identified by GC/MS and /or GLC analyses. The flavonoids were isolated from the ethyl acetate fraction and identified as naringenin, 7,3',4'-trimethoxy quercetin and lucenin-2 in addition to p-coumaric acid. The structure of these compounds were established using different spectroscopic techniques. The antioxidant activity of different extracts and fractions (pet. ether, fatty alc., fatty acids, 70% methanol, chloroform and ethyl acetate) were tested using a DPPH free radical assay. It was found that the 70% methanol extract and ethyl acetate fraction exhibited remarkable activity (88.43 and 78.39 % respectively).

Key words: *Rosaceae*, *Prunus avium*, seed oil, flavonoidal constituents and antioxidant activity.

INTRODUCTION

Prunus avium L.(*PA*, Sweet cherry, family Rosaceae) has been cultivated since ancient times, but recently have attention due of its health benefits attributed to the presence of antioxidants and pain-relieving bioactive components [1-2]. Many authors have been studied the different biological activities and chemical constituents of PA.

The seeds of *Prunus cerasoides* are used for the treatment of stone in the kidney [3], while the stem is reported to be antipyretic, refrigerant and useful in vomiting, thirst, Asthma, leprosy and leucoderma [4].

Evangelosin, 1991[5] reported that PA kernel was found to contain protein of a high percentage of glutamic acid, arginine and aspartic acidbut methionine was the least one. While Bak *et al*., in 2010 [6] showed that, *Prunus cerasus* seed kernel oil contains vegetable oils including unsaturated fatty acids, oleic acids, alpha-tocopherol and tocopherol-like components. In addition to, the components of the solid fraction include polyphenols, flavonoids, vegetable acids, and pro- and anthocyanidins, which could have useful therapeutic values in the prevention of many diseases.

Straccia *et al.*, in 2012 [7] investigated the compositions of free fatty acids and phytosterols of *PA* seed oil samples which obtained by two extraction methods andanalyzed with GLC. They proved that, the unsaponifiables obtained by the supercritical fluid extraction (SFE) and Soxhlet extraction (SE), which contain β-sitosterol and campesterol. Also the differences in the fatty acid composition as follow: with the SE, 48.6% of saturated fatty acids (SFA), 17.74% of unsaturated acids (USFA), 32.75% of polyunsaturated (PUSFA). On the other hand, with SFE decreased the percentage of SFA (38.43%) infavor of larger amounts of USFA (19.93%) and PUSFA (36.04%). It was found

that the SFE is more efficient to obtain oil with high quality, without any degradation phenomena due to the solvent or heat. In both SE and SFE high percentages of arachidic acid (C20:0) linoleic acid (C18:2, ω-6) and nervonic acid (C24:1, ω-9), of marine origin, precursor of the neuronal cell membrane glycolipids, with a key role in the modulation of ion channels and membrane receptors, and therefore, it used as a neurotrophic factor in food supplements and nutraceuticals. Many flavonoidal compounds has been isolated from the seeds of *Prunus cerasoides* which were characterized as: tectochrysin, genistein, leucocyanadin, 5-methoxy naringenin 7- O - galactoside, genkwanin, prunetin, sakuranetin, 4'-O-glucoside and naringenin 4'-methyl ether-7-O- glucoside by Jangwan and different authors [8-10].

Monika [11] have been identified seven flavonol glycosides from the leaves of *Prunus serotina* as: hyperoside, avicularin, reynoutrin, quercetin-3-*O*-(6"-*O*-rhamnopyran)-glucoside, quercetin-3-*O*-(2"-*O*-rhamnosyl)-glucoside and quercetin-3-*O*-(2"-*O*-rhamnosyl)-galactoside as well isorhamnetin 3-*O*-(6"-*O*-rhamno-glucoside).

Larysa *et al*., in 2016 [12] determined the most important components in *P. padus* fruits using HPLC which were characterized as : cyanidin-3-*O*-glycoside, cyanidin-3-*O*-rutinoside, caffeic acid , rutin and quercetin.

The aim of the present work was to study the lipid (oil, unsaponfiables, fatty acids and acetone insoluble fraction) and flavonoidal components, in addition to evalution of antioxidant activity of *PA* seeds.

MATERIALS AND METHODS

Plant material

Sweet cherries (*PA* of deep red colour) were purchased from Giza vegetables and fruits market in June 2015. The cherries were selected by eliminating unripe and too ripe fruits, with lesions and without stalk. Prior to processing, the seeds of the fruit were manually separated from the pulp, dried in shade and grinding to fine powder. The seeds were kindly, authenticated in the National Centre of Agriculture Research - Giza, Egypt.

Extraction of the oil and lipid constituents

About 1.25 kg of dried powdered seeds were extracted with petroleum ether (b.r. 40-60 °C) in a Soxhlet apparatus. The pet ether extract filtered, dried over anhydrous sodium sulphate and evaporated *in vacuo* at 40 °C till dryness to give an yellow oily residue (2.6% v/w) and divided to three parts, the 1st one was analyzed as it is using GC/MS analysis. The 2nd one (About 5 g) was dissolved in hot acetone, left overnight and filtrate the acetone insoluble fraction. The filtrate was freed from acetone and subjected to saponification process to afford the unsaponifiable materials and fatty acid methyl esters.

Extraction and isolation of flavonoidal components

About 1.125 kg of defatted powdered seeds were extracted with chloroform in a Soxhlet for 24 hrs. followed by maceration with methanol (70%, 3 x 2.5L) .The combined methanol extract was evaporated *in vacuo* at 50 ºC till free from methanol and diluted with hot distilled water (800 ml). The aqueous methanolic was partitioned with ethyl acetate (400 ml x 3) and the combined solvents was dried over anhydrous sodium sulfate and evaporated till dryness.

The ethyl acetate fraction was found to contain four flavonoidal spots which were isolated by passing over sephadex LH-20 column (3 x 50) eluted with 90% aqueous methanol. The fractions containing these compounds in semipure form were polled together, after then were rechromatographed on many Sephadex columns to afford compound C-I and C-II in pure form as yellowish powder (11 and 9mg respectively). The fraction containing compounds C-III and C-IV was subjected to preparative TLC with cellulose plates developed with 20% acetic acid. Two bands were localized, scraped off, eluted with 95% methanol and further purified over sephadex LH-20 column, eluted with 95% methanol to afford compounds C-III and C-IV in a pure form (4mg and 7 mg respectively).

Qualitative and quantitative characterization of oil

The oil of *PA* seeds was characterized by gas chromatography mass spectrometry. The GC-Ms analysis of the oil sample was carried out using gas chromatography–mass spectrometry instrument stands at the Laboratory of medicinal and aromatic plants, National Research Center, Egypt, with the following specifications. Instrument: a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a THERMO mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC-MS system was equipped with a TR-5MS column (30 m x 0.32 mm i.d., 0.25 μm film thickness). Analyses were carried out using helium as carrier gas at a flow rate of 1.3 mL/min at a split ratio of 1:10 and the following temperature program: 80 C for 1 min; rising at 4 C/min to 300 C and held for 1min. The injector and detector were held at 220 and 200 C, respectively. Diluted

samples (1:10 hexane, v/v) of 1 μL of the mixtures were always injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 40-450.

Qualitative and quantitative analyses: Most of the compounds were identified using two different analytical methods: (a) KI, Kovats indices in reference to n-alkanes (C9-C22); and (b) mass spectra (authentic chemicals, Wiley spectral library collection and NSIT library). The separated components of the essential oil were identified by matching with the National Institute of Standards and Technology (NIST) published [13] as shown in (Table1).

GC/MS analysis of acetone insoluble fraction

This analysis was performed using the following conditions, instrument, MS Model 88 SW/HW rev. Column: DB-1 (cross linked methyl silicone gum, 3m, 0.2mm i.d., 0.33µm film). Temp.: source: 200°C, analyzer 220°C, carrier gas: helium 600cc/min., injection splitless. The results were summarized in (Table 2).

GLC analysis of unsaponifiable matters and fatty acid methyl esters

The GLC analyses were carried out using the following conditions; Instrument: Varian model3700 GC. Column for unsap.: 10% OV-101 on chromsorb W/HP, 80/100, (2m stainless steel, 0.25mm i.d.), Column for fatty acid methyl esters:15% DEGS on chromsorb W/AW, 80/100, (2m stainless steel, 0.25mm i.d.), Temp. for unsap:column:70°C up to 270°C, 4°C/min., injector: 280°C., Detector (FID):290°C. Temp. for fatty acid methyl esters: column:70°C up to 190°C, 4°C/min., injector: 240°C., Detector :280°C, Flow Rates for both of them :N_2 and H_2: 30 ml / min., Air: 300 ml /min. The data were tabulated in (Tables 3,4).

DPPH Assay

The antioxidant capacity of the *Prunus avium* extracts was measured using a DPPH method described by Sun *et al.*, [14] using the free radical 2,2-diphyenyl-picrylhydrazyl (DPPH), with some minor revisions. Aliquots (0.1 mL) of diluted extracts in DMSO were added to 1 mL of DPPH solution and the absorbance of the DPPH solution was determined at 520 nm after 30 min of incubation at room temperature [15]. Appropriate blanks (DMSO) and standard (trolox solution in DMSO) were used to compare the antioxidant capacity of *Prunus avium* extracts. All measurements were done in triplicate.The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation :

$$\% \text{ RSA} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} X100$$

RESULTS AND DISCUSSION

Table 1. GC/MS data of *PA* oil

Peak No.	R_t(min.)	Area %	Compounds
1	5.29	1.06	Pentanal, 2-methyl
2	6.98	1.27	2-Heptenal, (Z)
3	11.98	2.32	Nonanal
4	16.07	2.50	2,4-Nonadienal
5	17.31	0.78	1-Dodecene
6	17.58	2.17	2-Decanynoic acid
7	18.35	3.94	1,4-Epoxycyclohex-2-ene
8	18.73	2.55	2,4-Decadienal
9	19.56	9.32	2,4-Decadienal,(E,E)
10	27.95	0.81	Heptadecane, 2,6,10,15-tetramethyl
11	42.92	2.05	Hexadecanamide
12	46.77	11.52	9-Octadecenamide
13	47.34	1.82	Octadecanamide
14	49.29	2.55	Hexadecanoic acid, 2,3-dihydroxypropyl ester
15	50.01	0.96	Phthalic acid, isodecyloctyl ester
16	54.75	12.36	13-Docosenamide, (Z)- =(Erucylamide)
17	55.01	1.33	Squalene
18	56.58	1.92	Dotriacontane
19	59.88	5.87	Tetratetracontane
20	63.17	18.57	γ-Sitosterol
21	63.43	2.32	Methylcommate-B
22	64.10	6.04	Lupenone
23	64.55	0.80	Lupeol
24	65.16	4.60	Cholesterone

According to Table 1, the results of GC-MS analysis of the oil of *PA* seeds showed that, the main components were:γ-sitosterol (18.57%), erucylamide(12.36%), 9 octa-decenamide (11.52%), 2,4-decadienal,(E,E) (9.32%), lupenone (6.04%), tetratetra-contane (5.87%), and cholesterone (4.60%).These data proved the presence of amide compounds with a high precentage (23.88%). In addition to, a sterol fraction constitute (23.17%), this the first report about the GC/MS of *PA* seeds oil.

Table 2. GC/MS of *PA* acetone insoluble fraction

Peak No.	R_t(min.)	Rel. %	Compounds
1	5.40	30.35	Octadecanol $C_{18}H_{36}O$
2	37.64	8.88	Docosanol $C_{22}H_{44}O$
3	55.62	55.27	Di 2- ethylhexyl phthalate
4	63.13	5.50	Pregnan-11-one

The results of GC/MS of acetone insoluble matters of *PA* in table 2 proved that, it is a mixture of fatty alcohol and phthalate: octadecanol (30.35 %), docosanol (8.88 %), di 2- ethylhexyl phthalate (55.27 %) beside pregnan-11-one (5.5 %).

Table 3. GLC data of *PA* unsap. fraction

Peak no.	R_t(min.)	Area %	Compounds
1	2.35	6.54	Hexane, n-C 6
2	3.28	6.50	Heptane, n-C 7
3	4.63	3.43	Octane, n-C 8
4	5.19	4.23	Nonane, n-C 9
5	6.77	2.29	Decane, n-C 10
6	8.33	8.19	Undecane, n-C 11
7	9.10	5.02	Dodecane, n-C 12
8	9.64	3.30	Tridecane , n-C 13
9	11.22	16.65	Tetradecane , n-C 14
10	12.06	28.77	Pentadecane , n-C 15
11	13.55	1.52	Hexadecane , n-C 16
12	14.63	2.54	Heptadecane, n-C 17
13	14.45	2.51	Octadecane, n-C 18
14	16.82	1.19	Nonadecane, n-C 19
15	17.94	0.86	Eicosane, n-C 20
16	19.97	1.25	Docosane , n-C 22
17	24.08	0.97	Hexacosane, n-C 26
18	29.59	2.58	Cholesterol
19	33.86	1.66	Stigmasterol

The data in Table 3 of the unsap. fraction of *PA* seeds, revealed the presence of a mixture of hydrocarbons from C_6 to C_{26} representing (95.76 %), in which C_{15}(28.77 %) was the main hydrocarbon in addition to a sterol fraction in which cholesterol (2.58 %) and stigmasterol (1.66 %) were present.

Table 4. GLC data of FAME fraction of *PA*

Peak No.	R_t(min.)	Rel.%	Compounds
1	15.11	22.50	Palmitic acid, n-C 16(0)
2	17.16	10.73	Margaric acid, n-C 17(0)
3	18.10	6.28	Stearic acid, n-C 18(0)
4	18.50	53.73	Oleic acid, n-C 18(1)
5	19.80	6.55	Linoleic acid, n-C 18(2)

The GLC analysis of FAME of the *PA* seeds (Table 4) proved the presence of a mixture of 5 fatty acids (FA), represented 99.97 % of the acid. It was found that this oil is rich in unsaturated fatty acids, which constitute 60.28 % of the total fatty acids. Among the unsaturated fatty acids, oleic acid is the major constituent and forms 53.73 % of the total composition. These data were in accordance with that reported by Bak *et al.*, in 2010 [6] where they stated that, *P. cerasus* seed kernel oil contain oleic acid in range of 38.6-53.2%.

Identification of flavonoids

7,3',4'-trimethoxyquercetin (1): Yellow powder, It has R_f value of 0.85 upon using BAW. It gave yellow colour under UV-light, no change on exposure to ammonia vapors and changed to greenish yellow when sprayed with $AlCl_3$ reagent using cellulose plates. The EI/MS displayed a molecular ion peak M^+ at *m/z*(%): 344(17), M^+ - CH_3, 329(5), M^+ - CHO, 315(8),301(4) due to the quercetin moeity-1, 165(40)for A_1^+ fragment. The ^{1}H-NMR (DMSO-d6) showed signals at δ in ppm =7.50 (1H, d, J =2Hz, H-2'), 7.22 (1H,dd, J =2.0, 8.4 Hz, H-6'), 7.08 (1H, d, J =8.4Hz H-5'), 6.80 (1H,d,J =2.0 Hz, H-8), 6.53 (1H,d, J =2Hz, H 6), 3.83, 3.86, 3.90 (each 3H, s, OMe)[16].

Lucenin-2 (2): This compound was isolated as a yellowish powder, appered as a purpule spot changed into greenish yellow by ammonia vapour and became yellow with NA reagent. It's behavoir on paper proved it's glycosdic nature where it has R_f =0.63 in 15% AcOH and 0.31 in BAW(3:1:1). The UV spectrum in methanol substantiated that, it is a flavone type structure (band-I at λ_{MeOH}=345 nm), the presence of dihydroxy groups at C-3' and C-4' was proved through $AlCl_3$/HCl and NaOAc/H_3BO_3 spectra and NaOAc spectrum displayed band-II at λ =270 nm with a bathochromic shift from methanol spectrum =15 nm which means the presence of a free hydroxy group at C-7.

The EI-mass spectrum displayed M^+ at *m/z*=610 which fited with the molecular formula $C_{27}H_{30}O_{15}$. The compound was found to resist theacid hydrolysis and responsed to the enzymatic hydrolysis with β- glucosidase affording glucose as a sugar and luteolin as an aglycone [17].

Naringenin (3): The UV data of this compound proved that, it is a flavanone type where it displayed band-II as a sholder at 326 nm; the other data with the other shift reagent proved the presence of free OH groups at C5,7,4' with no ortho dihydroxy system [18,19]. The EI- MS of the compound displayed a molecular ion peak M^+ at *m/z*=272(52) which correspond to the molecular formula $C_{15}H_{12}O_5$. Other peaks at 271(45)[M^+ -1], 153(100)[A_1+l] and 120(60)[B_1]$^+$confirm the structure of the compound as naringenin.The NMR data were shown in Table 5.

Table 5. ^{1}H and ^{13}C-NMR data of compound 3.

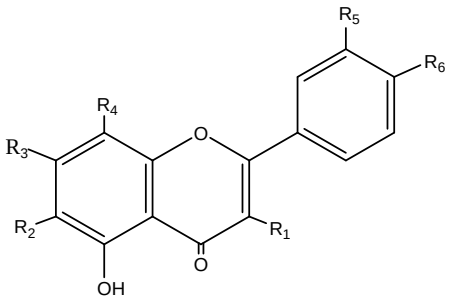
Carbon No.	δin ppm	
	^{1}H	^{13}C
2	5.46,dd,J=12.0and4.0,H-2	78.7
3	2.66,dd, J=14, 4Hz,H-3e,3.20,dd, J= 14, 12Hz,H-3a	42.6
4	-	196.1
5	-	163.5
6	5.94, 1H,s,H-6	96.7
7	-	166.7
8	5.94, 1H,s,H-8	95.6
9	-	163.4
10	-	102.5
1'	-	128.8
2'	7.37, 1H,d,J=8.5,H-2'	128.4
3'	6.82,1H,d,J =8.5,H 3'	115.4
4'	-	157.7
5'	6.82, 1H,d,J =8.5,H-5'	115.9
6'	7.37, 1H,d,J=8.5,H-6'	129.1

***p*-Coumaric acid (4):** This compound was isolated as a whitish crystals and it's UV spectrum in MeOH exhibited two bands at 310nm and 271nm. EI-MS displayed a molecular ion peak M^+ at *m/z*=164 which correspond to the molecular formula $C_9H_8O_3$. Another important peaks at 147(M^+- OH), 119(M^+ - COOH) and 92 (M^+ - CH_2=CHCOOH)[20]. The NMR data were in Table 6.

Table 6. ^{1}H and ^{13}C-NMR data of compound 4.

Carbon No.	δ in ppm	
	^{1}H	^{13}C
1	-	127.1
2	7.52, H,J=8.2 H-2	131.7
3	6.7, H, J=8.3 H-3	117.0
4	-	161.1
5	6.7, H, J=8.3 H-5	117.0
6	7.52, H,J=8.2 H-6	131.7
C=O	-	166.7
Cα	6.52, 1H, d, J=16	115.2
Cβ	7.64, 1H, d, J=16	165.1

All the structure of the isolated compounds were shown in Fig. 1.



*1- R_1=OH, R_2,R_4=H, R_3,R_5,R_6=OMe (*7,3',4'-trimethoxyquercetin)
*2- R1,R3,R5,R6= OH, R2,R4=C-glucose (*Lucenin-2)
3- Naringenin 4- p-Coumaric acid

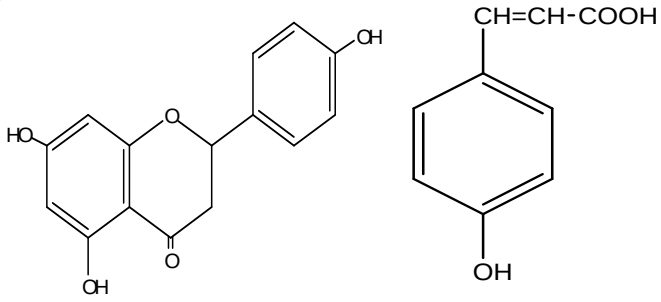


Fig. 1. The structure of the isolated compounds from PA seeds

DPPH assay

Results are expressed as radical scavenging activity (% RSA) as shown in Table 7 which proved that, the sample with the higher percentage has higher scavenging capacity. It was found that the methanol extract and ethyl acetate fraction exhibited remarkable activity (88.43 and 78.39 % respectively), when compared with oxidative potential of reference control (Trolox 97.3 %). This could be due to the nature of polyphenolic contents. These data were in accordance with that reported by Hasim and Serkan in 2011[21]. While the chloroform extract and pet. ether extract exhibited moderate activity and the fractions of fatty alcohol, unsap. and fatty acids showed the lowest activity.

Table 7. Antioxidant activity of *Prunus avium* seeds extracts

Samples	Mean absorption	*% DPPH Inhibition ± S.D.
Trolox	0.013	97.3±0.45
Fatty alcohol	0.294	36.90 ±0.21
Un sap.	0.320	31.32±0.42
Fatty acid	0.252	45.77± 0.45
Petroleum ether extract	0.211	54.79±0.44
Chloroform extract	0.165	64.44±0.87
Ethyl acetate extract	0.100	78.39±0.65
Methanol extract	0.053	88.43±0.86

Data are mean ±SD values.
**Expressed as % of neutralised DPPH free radicals.*

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